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# Characterization of Calcineurin in Human Neutrophils

## INHIBITORY EFFECT OF HYDROGEN PEROXIDE ON ITS ENZYME ACTIVITY AND ON NF-κB DNA BINDING*

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**We describe here a specific calcineurin activity in neutrophil lysates, which is dependent on $Ca^{2+}$, inhibited by trifluoroperazine, and insensitive to okadaic acid. Immunoblotting experiments using a specific antiserum recognized both the A and B chains of calcineurin. Neutrophils treated with cyclosporin A or FK 506 showed a dose-dependent inhibition of calcineurin activity. The effect of oxidant compounds on calcineurin activity was also investigated. Neutrophils treated with hydrogen peroxide ($H_2O_2$), where catalase was inhibited with aminotriazole, exhibited a specific inhibition of calcineurin activity. However, the addition of reducing agents to neutrophil extracts partially reversed the inhibition caused by $H_2O_2$. A similar inhibitory effect of $H_2O_2$ on calcineurin activity was observed to occur in isolated lymphocytes. This is the first demonstration that redox agents modulate calcineurin activity in a cellular system. In addition, electrophoretic mobility shift assays revealed that lipopolysaccharide-induced activation of NF-κB in human neutrophils is inhibited by cell pretreatment with $H_2O_2$ in a dose-dependent manner. These data indicate that calcineurin activity regulates the functional activity of lipopolysaccharide-induced NF-κB/Rel proteins in human neutrophils. These data indicate a role of peroxides in the modulation of calcineurin activity and that the $H_2O_2$-dependent NF-κB inactivation in neutrophils occurs in concert with inhibition of calcineurin.**

Calcineurin (CN)[1] has recently been established as a key enzyme in the signal transduction cascade leading to T cell activation (1–4), and an important regulator of transcription factors such as NF-AT, NF-κB, and AP-1, which are involved in the expression of a number of important T cell early genes, *i.e.* interleukin-2, tumor necrosis factor-α, and interleukin-2R (5–8). CN, also known as phosphatase 2B, is a calcium/calmodulin-dependent serine/threonine phosphatase (9–11) and is composed of the following two subunits: a 59-kDa catalytic subunit (CNA), which contains a calmodulin-binding domain and an autoinhibitory region, and a 19-kDa intrinsic calcium-binding regulatory subunit (CNB) (12–14). Human CN possesses a Fe-Zn active center. The assignment of stoichiometric amounts of $Zn^{2+}$ and $Fe^{3+}$ in the CNA center is based on atomic absorption experiments (15). The same assignments were made for the di-metal site in the structure of some CNA (16).The central role of CN in T cell signaling was appreciated by its identification as the target of the immunosuppressive drugs cyclosporin A (CsA) and FK 506 (1–4). The phosphatase activity of CN is inhibited by either drug when complexed to intracellular binding proteins (immunophilins), *i.e.* CsA to cyclophilin and FK 506 to the FK 506-binding protein 12 (FKBP12), respectively. Neither drug nor immunophilin alone bind to or affect the activity of CN (1). This phosphatase is expressed ubiquitously in eukaryotic cells. In mammals, CN is most abundant in the brain (17) but has also been detected in T cells (1–4). On the other hand, it is known that NF-AT-mediated transactivation depends on the CN activity (18, 19). Other findings suggest that NF-κB activity is also under CN control (20–22).

In neutrophils, only indirect evidence has been presented on the occurrence of the phosphatase CN. The treatment of these cells with inhibitors of CN (*e.g.* CsA and FK 506) inhibited the neutrophils chemokinesis on vitronectin matrix (23, 24). Furthermore, intracellular calcium and CN regulate neutrophil motility on vitronectin through a receptor identified by antibodies against the integrins αv and $\beta_3$ (25, 26). The first purpose of the present work was to assess the presence of CN in neutrophils using as a substrate a specific peptide corresponding to the phosphorylation site of the RII subunit of cyclic AMP-dependent protein kinase.

Additionally, reactive oxygen intermediates (ROI) have been implicated in mediating signal transduction by a variety of stimuli in lymphoid cells, and transcription factors seem to be responsible for the inducible expression of a number of genes in response to oxidative stress (27, 28). In this context, the addition of $H_2O_2$ to the culture medium has been shown to activate NF-κB (29). Hydroxyl radicals produced from $H_2O_2$ cannot function as diffusible intracellular messengers, since they can react with the nearest molecule in a nonspecific fashion. A

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[1] The abbreviations used are: CN, calcineurin; NF-AT, nuclear factor of activated T cells; NF-κB, nuclear factor κB; CsA, cyclosporin A; CNA, calcineurin A subunit; CNB, calcineurin B subunit; ROI, reactive oxygen intermediates; $H_2O_2$, hydrogen peroxide; pV(phen), sodium oxodiperoxo(1,10-phenanthroline)vanadate(V); HBSS, Hanks' balanced salt solution; BSA, bovine serum albumin; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; PDTC, pyrrolidine dithiocarbamate; AMT, 3-amino-1,2,4-triazole; PAGE, polyacrylamide gel electrophoresis; DFP, diisopropyl fluorophosphate; $H_2$DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; EMSA, electrophoretic mobility shift assay; LPS, lipopolysaccharide.

more suitable ROI messenger would be the less reactive $H_2O_2$. However, arguments against ROI involvement in NF-κB activation have been published (30–32), and despite the fact that phorbol 12-myristate 13-acetate-dependent NF-κB stimulation is cancelled by antioxidants, it has been recently shown that phorbol 12-myristate 13-acetate does not increase intracellular ROI (33). Peroxide-mediated stimulation of NF-κB appears to be cell line-specific, since *N*-acetylcysteine, an antioxidant, elicited up-regulation of NF-κB binding activity in monocyte-derived macrophages (34). Moreover, NF-κB is not the only nuclear factor whose activity is altered by $H_2O_2$. In a fashion opposite to that observed for NF-κB, NF-AT has been shown to be actively suppressed by $H_2O_2$ in Jurkat T cells (35). Recently, the development of stabilized peroxovanadium compounds has provided the opportunity to more fully characterize the action of oxidants within the cell (36). Treatment of lymphocytes with sodium oxodiperoxo(1,10-phenanthroline)vanadate (V), pV-(phen), results in a large increase in intracellular oxidation, which correlates with a strong induction of cellular tyrosine phosphorylation and activation of kinases. The same authors (37) have documented that antioxidant treatment does not prevent the activation of NF-κB by pV(phen). Therefore, we have also used pV(phen) as another tool to study the effect of oxidative stress on calcineurin activity.

In summary, previous data suggest that, first, CN modulates the DNA binding activity of essential transcription factors (*e.g.* NF-AT and NF-κB), and second, ROI regulates positively or negatively those transcription factors. However, a link between both signals, that is CN and ROI, is lacking. In this paper we address this question and the implications of the ROI as universal messenger to activate NF-κB.

## EXPERIMENTAL PROCEDURES

*Cell and Reagents*—Neutrophils were isolated from fresh heparinized blood of healthy human donors by dextran sedimentation, followed by Ficoll-Paque gradient centrifugation and hypotonic lysis of residual erythrocytes as indicated (38). Neutrophils were washed twice in Hanks' balanced salt solution (HBSS), suspended at a density of $1 \times 10^7$ cells/ml in HBSS supplemented with 0.1 mg/ml BSA, and maintained at 37 °C in an atmosphere of 5% $CO_2$ and 95% $O_2$ for 1–2 h. Peripheral blood lymphocytes were obtained from heparinized venous blood of normal volunteers by Ficoll-Paque centrifugation.

Dextran T-500 was obtained from Pharmacia Biotech (Barcelona, Spain). Ficoll-Paque, HBSS, and RPMI 1640 were obtained from BioWhittaker (Verviers, Belgium). CsA and FK 506 were kindly provided by Dr. S. F. Borel (Sandoz Ltd., Basel, Switzerland) and Fujisawa GmbH (München, Germany), respectively. Chemicals were of analytical grade from Merck (Darmstadt, Germany). Dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF) were obtained from Boehringer Mannheim (Barcelona, Spain). Bovine serum albumin (BSA), okadaic acid, trifluoroperazine, pyrrolidine dithiocarbamate (PDTC), hydrogen peroxide (30% v/v), 3-amino-1,2,4-triazole (aminotriazole, AMT), soybean trypsin inhibitor, leupeptin, aprotinin, Nonidet P-40, and goat anti-rabbit IgG conjugated to horseradish peroxidase were purchased from Sigma (Madrid, Spain). Rabbit anti-bovine calcineurin IgG was kindly provided by C. B. Klee. The synthetic peptide used as a substrate for calcineurin was purchased from Peninsula Laboratories (Bellmont, CA). pV(phen) was synthesized as described previously (37). [γ-$^{32}$P]ATP was obtained from NEN Life Science Products. 2-Mercaptoethanol, SDS, acrylamide, *N,N′*-methylene-bisacrylamide, Coomassie Brilliant Blue R-250, and blotting nitrocellulose membranes were purchased from Bio-Rad. Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and diisopropyl fluorophosphate (DFP) were purchased from Serva (Madrid, Spain), and 4-iodophenol was from Aldrich (Madrid, Spain). Molecular weight standards (Rainbow markers) was obtained from Amersham Corp. (London, UK); Sephadex G-25 was from Pharmacia (Barcelona, Spain); and double-stranded oligonucleotide probe (5′-AGTTGAG GGACTTTCC CAGGC-3′) containing NF-κB sites was from Boehringer Mannheim Gmbh (Mannheim, Germany). 2′,7′-Dichlorodihydro-fluorescein diacetate ($H_2$DCFDA) was purchased from Molecular Probes (Leiden, The Netherlands).

*Crude Neutrophils Extract*—Untreated neutrophils ($1 \times 10^8$ cells/ml) were lysed for 30 min on ice in 500 μl of buffer A (50 mM Tris, pH 8.0, 0.5% Triton X-100, 150 mM NaCl, 50 μg/ml PMSF, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and disrupted by sonication. The homogenate was centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant fluid (crude extract) was separated from low molecular weight material by passage through a 0.5 × 10-cm Sephadex G-25 column equilibrated with 50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.1% β-mercaptoethanol, and a mixture of protease inhibitors as above (39). Fractions containing most of the $A_{280\ nm}$ material eluting with the void volume of the column were pooled and used to measure CN activity.

*Cell Treatments and Lysis*—Immunosuppressive agents were dissolved in dimethyl sulfoxide ($Me_2SO$) at a concentration 1000-fold higher than that used for cell treatments. Neutrophils ($7 \times 10^6$ cells/ml) were suspended in 1 ml of HBSS supplemented with 0.1 mg/ml BSA in microcentrifuge tubes, 1 μl of $Me_2SO$ or CsA or FK 506 was added, and the cells were incubated at 37 °C for 2 h. For experiments with $H_2O_2$ and other stimuli, neutrophils were incubated at 37 °C. The incubation times and concentration of agents are indicated in the figure legends. After incubation, the cells were washed once with 1 ml of HBSS on ice and lysed in 60 μl of buffer B (50 mM Tris, pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 0.5 mM dithiothreitol, 50 μg/ml PMSF, 50 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 10 μg/ml aprotinin) and disrupted by sonication. Cell debris was removed by centrifugation at 4 °C for 10 min at 12,000 × *g*, and supernatant was used as the source of CN.

*Calcineurin Phosphatase Assay*—CN phosphatase activity was measured using an assay adapted from Hubbard and Klee (40), basically as described (22). Neutrophils ($7 \times 10^6$ cells/ml) were incubated for 2 h at 37 °C in the presence or absence of drugs as indicated in the text. Reaction mixtures containing 2 μM $^{32}$P-labeled phosphopeptide, 500 nM okadaic acid (added to inhibit PP-1A and PP-2A type phosphatase activities), and 20 μl of cell lysate (about 80 μg of protein) were incubated in a total volume of 60 μl of assay buffer C (20 mM Tris, pH 8.0, 100 mM NaCl, 6 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 mM $CaCl_2$ or 5 mM EGTA, as indicated in the figures) for 15 min at 30 °C. After this time, reactions were terminated by the addition of 0.5 ml of 100 mM potassium phosphate buffer, pH 7.0, containing 5% trichloroacetic acid. The reaction mixture was passed through a 500-μl column of activated Dowex cation-exchange resin, and free inorganic phosphate was quantitated in the eluate by scintillation counting. It was verified that at 15 min of incubation time the assay was linear. Assays were performed in triplicate, and the counts/min measured in blank assay lacking cell lysate were subtracted. Data are expressed as the number of picomoles of $^{32}PO_4$ released in 15 min per mg of protein.

*$^{32}$P-Labeled Phosphopeptide*—The synthetic peptide (Asp-Leu-Asp-Val-Pro-Ile-Pro-Gly-Arg-Phe-Asp-Arg-Arg-Val-Ser-Val-Ala-Ala-Glu), corresponding to a segment of the RII subunit of cAMP-dependent kinase (41), was phosphorylated on the unique serine residue by the catalytic subunit of cAMP-dependent protein kinase using [γ-$^{32}$P]ATP, essentially as described (40), and used as phosphatase substrate. The specific activity of fresh preparations of $^{32}$P-labeled phosphopeptide was about 500 μCi/μmol peptide.

*Western Blot Analysis*—Cells ($7 \times 10^6$ cells/ml) were lysed for 30 min on ice in 100 μl of buffer A (see above). Lysates were clarified by centrifugation at 4 °C for 2 min at 12,000 × *g*. Protein concentrations in the lysates were determined by the Bradford method (42), using BSA as a standard. For the Western blot analysis of CN subunits A and B, neutrophil lysates were subjected to 12.5% SDS-PAGE followed by electroblotting onto nitrocellulose using the Bio-Rad Mini-blotting apparatus. Filters were blocked for 1 h in TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) containing 3% BSA. Furthermore, they were rinsed twice with TBS containing 0.1% Tween 20 (TBST), and they were incubated overnight with rabbit anti-bovine calcineurin IgG diluted 1:1000 in TBST. After three washes in TBST, the filters were incubated for 90 min with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, final dilution). Filters were then washed twice with TBS and then twice with TBST for 10 min each time. The bound secondary antibody was detected by enhanced chemiluminescence (43). Briefly, the membranes were incubated for 1 min in 10 ml of fresh luminescent reagent solution, composed of 10 mM Tris-HCl, pH 8.5, 2.25 mM luminol, 0.015% (v/v) $H_2O_2$, and 0.45 mM 4-iodophenol, the latter acting as an enhancer of the chemiluminescence reaction (44). These concentrations of luminol, $H_2O_2$, and 4-iodophenol were determined to be optimal for maximum light production.[2] Luminol and 4-iodophenol were freshly prepared in 10 ml of 10 mM Tris-HCl, pH 8.5. Luminol was previously dissolved in 50 μl of 1 M NaOH. The use of $Me_2SO$ as solvent should be

[2] M. Carballo and F. Sobrino, unpublished data.

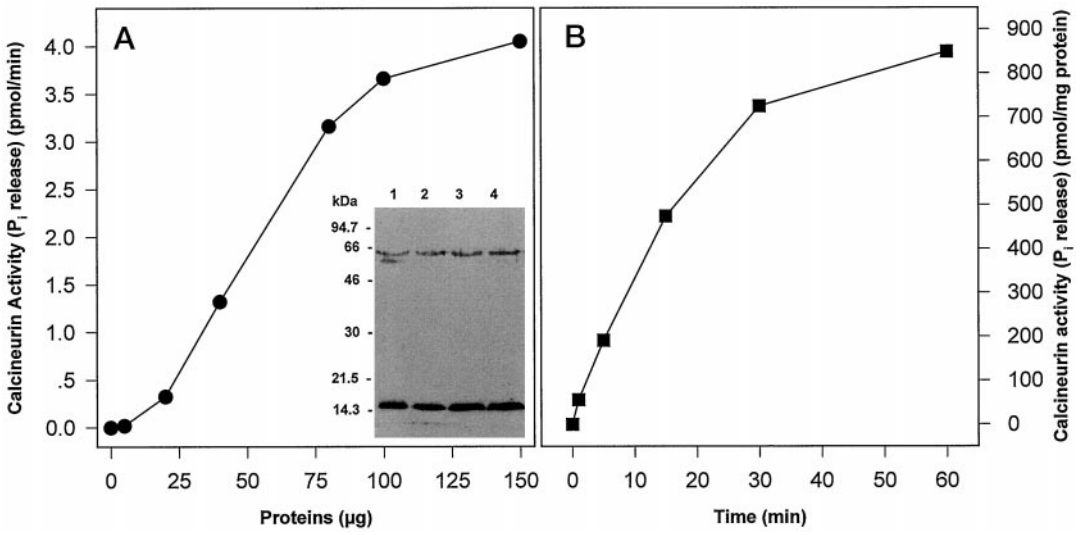


FIG. 1. **Specific dephosphorylation of a synthetic peptide substrate by crude neutrophil lysates.** Calcineurin phosphatase activity was measured as indicated under "Experimental Procedures." Reaction mixtures contained 2 $\mu$M $^{32}$P-labeled phosphopeptide and 500 nM okadaic acid in a total volume of 60 $\mu$l of assay buffer C. *A,* calcineurin activity was assayed using increasing amounts of neutrophil lysates during 15 min of incubation at 30 °C. *B,* calcineurin activity was assayed during the indicated times with 50 $\mu$g of protein from neutrophil lysates. *Inset*, immunoblotting analysis of calcineurin expression. Proteins were resolved by SDS-PAGE (12% polyacrylamide gel), transferred to nitrocellulose, and probed with an antiserum that recognizes both the A and B chains of calcineurin. *Lane 1*, blood human lymphocytes (50 $\mu$g of protein); *lanes 2–4*, crude neutrophil lysates (25, 50, and 100 $\mu$g of protein, respectively). Size markers are indicated on the *left*.

avoided since in alkaline/$Me_2SO$ conditions, luminol autoxidizes with emission of intense luminescence (45). After 1 min of incubation, the membranes were placed on paper filter, covered with Saran Wrap, and exposed to x-ray films (X-Omat, Eastman Kodak Co.) in the dark for 1–5 min.

*Electrophoretic Mobility Shift Assays (EMSA)*—Human neutrophils whose viability exceeded 98% after 3 h in culture, as determined by trypan blue exclusion, were resuspended at a density of $5 \times 10^6$ cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells were then incubated at 37 °C under a 5% $CO_2$ atmosphere for 3 h, with occasional shaking in the absence or presence of 1 $\mu$g/ml LPS, 1 $\mu$g/ml CsA, or $H_2O_2$ at the concentrations indicated in each experiment. Then nuclear extracts were prepared basically as described by McDonald *et al.* (46). With this purpose, cell suspensions were transferred into pre-cooled tubes containing an equivalent volume of ice-cold RPMI 1640 supplemented with DFP (2 mM, final concentration). After centrifugation at 4,000 $\times$ *g* for 1 min at 4 °C, the cells were resuspended in 100 $\mu$l of ice-cold relaxation buffer, consisting of 10 mM HEPES, pH 7.3, 30 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, and 0.5 mM DTT, supplemented with an antiprotease mixture composed of 2 mM DFP, 1 mM PMSF, 10 mM iodoacetamide, 1 mM benzamidine, and 10 $\mu$g/ml each of aprotinin, leupeptin, and captopril. Cells were disrupted by short sonication (1 s), and the lysates were spun at 3,000 $\times$ *g* for 10 min at 4 °C to pellet unbroken cells and intact nuclei. The pellets were resuspended in 100 $\mu$l of ice-cold relaxation buffer, again subjected to short (1 s) sonication, and respun as above to pellet intact nuclei. These were resuspended in 200 $\mu$l of relaxation buffer, and after new centrifugation, the nuclear pellet was resuspended in 25 $\mu$l of ice-cold relaxation buffer, additionally containing 10% (v/v) glycerol and 380 mM NaCl. Following a 20-min incubation on ice with occasional mixing, the samples were spun at 13,000 $\times$ *g* for 15 min at 4 °C, and the resulting supernatants were stored at −70 °C. EMSA was performed by using as a probe the double-stranded 22-base pair NF-$\kappa$B consensus oligonucleotide indicated above, which was labeled with DIG using the labeling kit from Boehringer Mannheim. The nuclear extracts (5 $\mu$g of protein) were assayed for $\kappa$B binding activity using the DIG gel shift assay kit (Boehringer Mannheim, GmbH). The reactions were performed in 15 $\mu$l of binding buffer (20 mM Tris-HCl, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 0.1% (v/v) Nonidet P-40, 6% glycerol) and allowed to proceed for 20 min at room temperature. For competition assays, binding reactions were performed in the presence of the unlabeled oligonucleotide (100-fold molar excess) for 20 min at room temperature. Supershift assays including anti-p50 and anti-p65 were carried out as described previously (46). The samples were finally electrophoresed on 5% polyacrylamide native gels at 4 °C in 0.25× TBE.

## RESULTS

*Calcineurin Is Present in Neutrophil Lysates*—CN is a well characterized phosphatase that plays an important role in T cell activation pathways (1–4). In this work we have characterized the presence of CN activity and immunoreactive CN protein in human neutrophils. In crude neutrophil lysates, specific CN activity was observed, and a linear appearance of product during the assay was obtained in the range of 20–75 $\mu$g of lysate proteins (Fig. 1*A*). The time course of CN activity is shown in Fig. 1*B*. Clearly, $^{32}$P release increased linearly along assay time until 15 min, and then a slow activity was found. Fig. 1 (*inset*) illustrates an immunoblotting analysis of CN expression in lymphocytes (*lane 1*) and different amounts of human neutrophil lysates (*lanes 2–4*). We used an antiserum that recognizes both the A (59 kDa) and B (19 kDa) chains of CN, confirming that both subunits are expressed in human neutrophils. As shown, neutrophil CNB migrates as a 16-kDa band in SDS-polyacrylamide gels, and it is detected along with a predominant CNA band migrating at 59 kDa. A third band, detected at 57 kDa in lymphocytes extracts, is probably a proteolytic fragment of CNA generated during preparation of the cell lysates. In some preparations of neutrophil lysates, a similar band of 55–57 kDa was also found (data not shown). Next experiments were addressed to analyze the regulation of CN activity in crude neutrophil lysates. Fig. 2 illustrates that the dephosphorylation of the CN-specific substrate peptide by crude neutrophil lysates was $Ca^{2+}$-dependent, as well as insensitive to okadaic acid, a potent and specific inhibitor of phosphatases 1A and 2A (reviewed in Ref. 47). When 500 nM okadaic acid was included in the assays, nearly all of the remaining phosphatase activity was $Ca^{2+}$-dependent and could be eliminated by substituting 5 mM EGTA for $Ca^{2+}$ (Fig. 2). In contrast, the okadaic acid-sensitive component was resistant to EGTA, which is consistent with the reported $Ca^{2+}$ independence of phosphatases 1A and 2A (17). CN activity was abrogated in nominally calcium-free medium and in the presence of a known inhibitor of calmodulin, trifluoroperazine (48). However, trifluoroperazine did not inhibit calcium-independent, okadaic acid-sensitive phosphatases from neutrophil lysates (Fig. 2). Taken

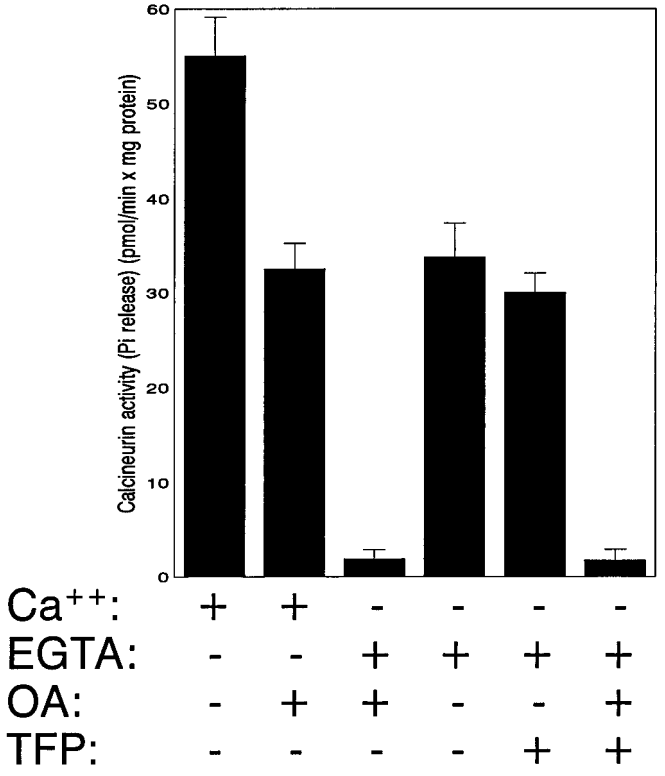


FIG. 2. **Effect of calcium, okadaic acid, and trifluoroperazine on calcineurin activity from crude neutrophil lysates.** Reaction mixtures contained 20 $\mu$l of crude neutrophil lysate (80 $\mu$g of protein), 2 $\mu$M $^{32}$P-labeled phosphopeptide, and 40 $\mu$l of assay buffer. Other additions were as follows: 0.1 mM $CaCl_2$, 5 mM EGTA, 500 nM okadaic acid (*OA*), or 200 nM trifluoroperazine (*TFP*), as indicated. CN activity was assayed during 15 min at 30 °C. Phosphatase activity is expressed as picomoles of phosphate released per min per mg of protein.

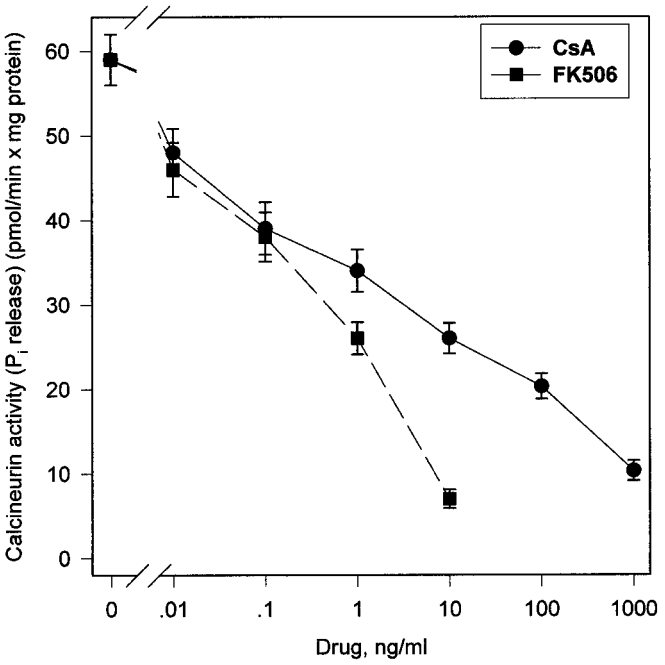


FIG. 3. **Calcineurin phosphatase activity in CsA and FK 506-treated neutrophils.** Neutrophils ($7 \times 10^6$ cells/ml) were incubated in 1 ml of incubation medium with the indicated doses of CsA and FK 506 for 2 h at 37 °C. Then the cells were harvested and washed, and the pellet was lysed in buffer B. Calcineurin activity was assayed in the cell lysates (80 $\mu$g of protein) as indicated under "Experimental Procedures." Phosphatase activity is expressed as picomoles of phosphate released per min per mg of protein. Three experiments were performed with similar results.

together, these data indicate that a specific $Ca^{2+}$/calmodulin-dependent phosphatase activity is present in the neutrophil lysates.

*Inhibition of Calcineurin Activity in Isolated Neutrophils Treated with Immunosuppressive Drugs*—As previously indicated (1–4), CsA and FK 506 can now be used as tools to elucidate the participation of CN on signal transduction processes. To assess whether treatment with these drugs inhibits CN activity, neutrophils were incubated with different concentrations of FK 506 and CsA for 2 h, and phosphatase activity was measured in cell lysates. Both agents effectively inhibited $Ca^{2+}$-dependent phosphatase activity, as shown in Fig. 3. These results indirectly suggested that the drug-sensitive phosphatase present in neutrophils is CN. Furthermore, in drug titration experiments both FK 506 and CsA inhibited CN activity in a concentration-dependent fashion. $IC_{50}$ values determined for CN inhibition were approximately 0.5 ng/ml for FK 506 and 5 ng/ml for CsA. This greater sensitivity to FK 506 than to CsA exhibited by neutrophil CN is similar to that previously described for lymphocyte CN (1–4).

*Effect of Oxidants on Calcineurin Activity*—As previously indicated, there is a clear relationship between the oxidative stress and activation or suppression of transcription factor activity (27–29). Since it has been shown that $H_2O_2$ suppresses the transcriptional activation of NF-AT (35) and that NF-AT is able to directly interact with CN (18, 19), we have explored whether $H_2O_2$ could alter CN activity. Fig. 4 illustrates that preincubation of neutrophils with $H_2O_2$ alone had no effect on this phosphatase. However, the preincubation of cells with AMT, an inhibitor of catalase, for 30 min and the further addition of $H_2O_2$ produced a clear decrease in CN activity. These data suggest that intracellular catalase in neutrophils (49) diminishes the effective concentration of exogenously added $H_2O_2$ and also indicate a potential protective role of catalase against CN inactivation. The preincubation of cells with AMT/$H_2O_2$ and PDTC, an antioxidant, partially ameliorated the inhibitory effect of $H_2O_2$ on CN activity. Neither AMT nor PDTC added by themselves had any effect on CN activity. The antioxidant action of PDTC against AMT/$H_2O_2$ inhibition was found to occur only when equimolar concentrations of $H_2O_2$ and PDTC were used; at 500–1000 $\mu$M $H_2O_2$ the preincubation with 100 $\mu$M PDTC was without effect (data not shown). A similar inhibitory effect of AMT/$H_2O_2$ on CN activity was observed to occur in isolated human lymphocytes. The treatment of these cells with 500 $\mu$M $H_2O_2$ or 50 $\mu$M pV(phen) for 1 h resulted in a marked decrease in CN activity (54 and 43% inhibition after $H_2O_2$ and pV(phen)-treatment, respectively). To explain this inhibitory effect of $H_2O_2$ on CN activity two possibilities may be raised: (i) $H_2O_2$ alters the CN polypeptide structure, and (ii) $H_2O_2$ damages CN prosthetic group structure. To test the first hypothesis, lysates from neutrophils previously incubated with AMT and $H_2O_2$ were analyzed by immunoblotting using a specific CN antibody. Fig. 5 shows that these agents did not alter the ability of the A and B subunits to be recognized by the anti-calcineurin antibody, suggesting that the CN protein structure remained intact. However, the modification of prosthetic groups associated with the catalytic center of CN remains a possibility. Previous data (50) describe a protective role of superoxide dismutase on partially purified CN from brain, suggesting that the environment redox can alter the structure of Fe-Zn center of CN in a reversible manner. In agreement with this possibility we have observed (Fig. 6) that the inhibition of CN by $H_2O_2$ in a neutrophil cell-free system can be partially reversed by the further addition of $Fe^{2+}$, DTT, or ascorbate, all of which act as reductants. A

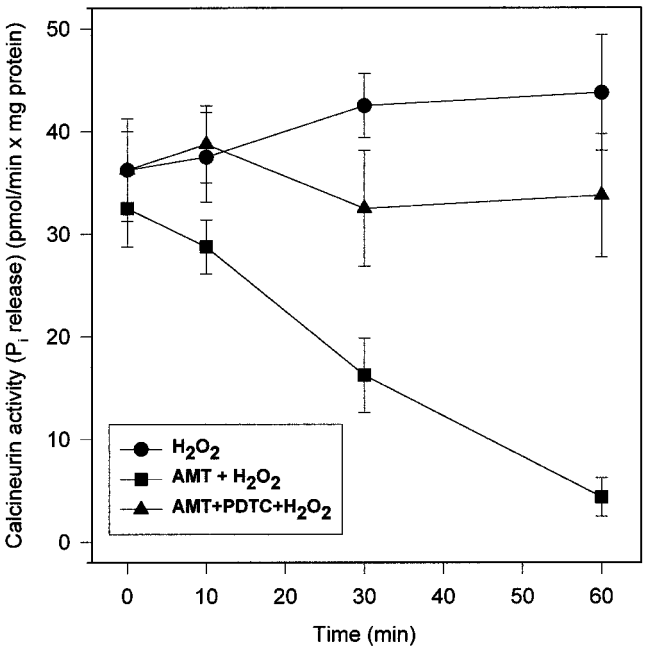


FIG. 4. **Inhibition of calcineurin activity in neutrophils treated with hydrogen peroxide.** Neutrophils ($7 \times 10^6$ cells/ml) were incubated at 37 °C under the following conditions: with 100 $\mu$M $H_2O_2$ alone (●); preincubated with 25 mM AMT for 30 min and then with 100 $\mu$M $H_2O_2$ (■); preincubated with 25 mM AMT for 30 min, then 100 $\mu$M PDTC for 10 min, and finally treated with 100 $\mu$M $H_2O_2$ (▲). The times of incubation with $H_2O_2$ are indicated. Incubation of cells was stopped at different times, and calcineurin activity was assayed in cell lysates (80 $\mu$g of protein) as indicated under "Experimental Procedures." Phosphatase activity is expressed as picomoles of phosphate released per min per mg of protein. Means ± S.E. values from three separate experiments performed in triplicate are presented.

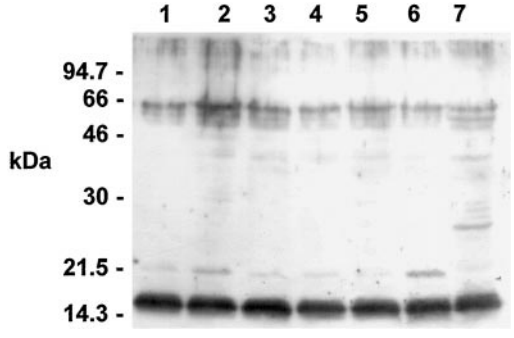


FIG. 5. **Immunoblot analysis of the stability of calcineurin structure.** Neutrophils ($1 \times 10^7$ cells/ml) were incubated under the following conditions: *lane 1*, no additions (control); *lane 2*, 0.5 mM $H_2O_2$ for 30 min; *lane 3*, 25 mM AMT for 30 min and then 0.5 mM $H_2O_2$ for 30 min; *lane 4*, 100 $\mu$M PDTC for 10 min and then 0.5 mM $H_2O_2$ for 30 min; *lane 5*, 25 mM AMT for 30 min and then 100 $\mu$M PDTC for 10 min; *lane 6*, 25 mM AMT for 30 min followed by 100 $\mu$M PDTC for 10 min and then 0.5 mM $H_2O_2$ for 30 min; *lane 7*, T cells without treatment. Proteins were resolved by SDS-PAGE (12% polyacrylamide gel), transferred to nitrocellulose, and probed with a polyclonal antiserum against calcineurin. The band at 28 kDa is probably a proteolytic fragment of calcineurin A generated during the preparation of cell lysates.

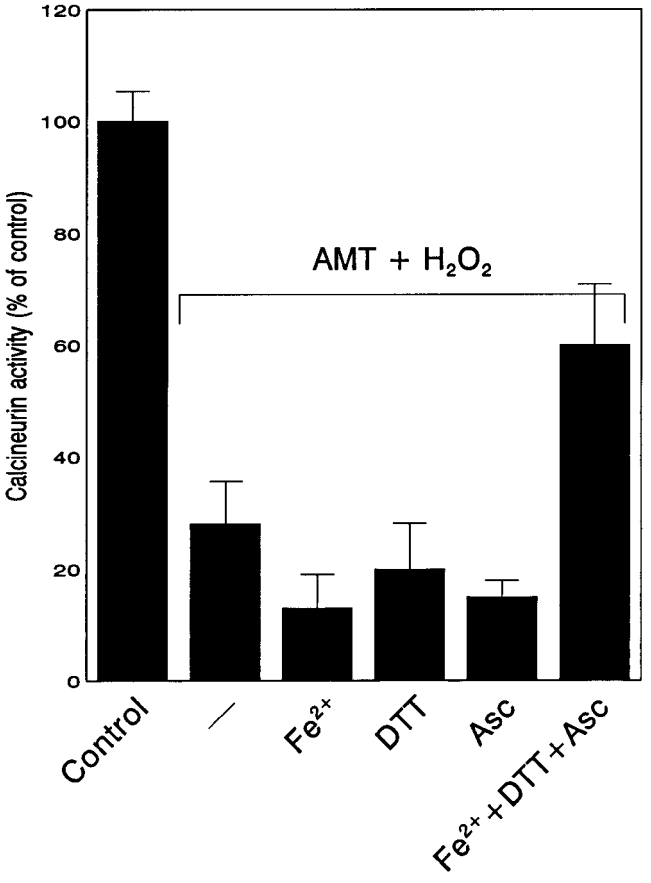


FIG. 6. **Reversibility by $Fe^{2+}$, ascorbate, and dithiothreitol of $H_2O_2$-inhibited calcineurin in neutrophil lysates.** Cell lysates (80 $\mu$g of protein) were incubated with 5 mM AMT for 5 min, followed with 100 $\mu$M $H_2O_2$ for 10 min. When present, 0.5 mM ferrous sulfate ($Fe^{2+}$), 5 mM DTT, or 5 mM sodium ascorbate (*Asc*) were added, either alone or altogether, for 20 min before the peptide substrate for the assay of calcineurin activity was added. Phosphatase activity is expressed in relation to the value obtained without cell treatment (*Control*).

previous report on the protective effect of these agents was documented in a partially purified CN assay activity from brain (50). Thus, our results are more consistent with a reversible modification of the catalytic center of CN elicited by $H_2O_2$.

$H_2O_2$ rapidly diffuses away from and into cells. In order to analyze the ability of neutrophils to internalize exogenous $H_2O_2$, intracellular oxidant levels were monitored by measuring the fluorescence of $H_2$DCFDA, a highly fluorescent probe sensitive to peroxides (51). Cells labeled with $H_2$DCFDA displayed a significant increase in DCF fluorescence upon incubation with $H_2O_2$ (154.5 ± 3.2 fluorescence units in 100 $\mu$M $H_2O_2$-treated cells *versus* 12.7 ± 1.1 units in control cells). In other experiments, the treatment of neutrophils with a variety of physiological and pharmacological stimuli revealed that agents that promoted an increase of intracellular $H_2O_2$ also induced a dose-dependent inhibition of CN activity in human neutrophils. Notably, both tumor necrosis factor-$\alpha$ (50 ng/ml) and interferon-$\gamma$ (50 units/ml) elicited an inhibition of CN activity of about 25% after 2 h of treatment. Increased times of tumor necrosis factor-$\alpha$ treatment (*e.g.* 8 h) resulted in an enhanced inhibitory effect (of about 35%) on CN activity. However, LPS (100 ng/ml), platelet-activating factor, or glucocorticoids were without effect on CN activity (data not shown).

To investigate whether the $H_2O_2$ effect was specific, we also analyzed the CN activity with a new peroxovanadium compound, pV(phen), which causes intracellular oxidative stress and induces strong protein tyrosine phosphorylation (37). The treatment of neutrophils with pV(phen) caused a dose- and time-dependent inhibition of CN activity (Fig. 7). After 60 min of incubation of the cells with 50 $\mu$M pV(phen), an inhibition of about of 60% was observed, with an apparent $IC_{50}$ of about 30 $\mu$M. Therefore, we provide evidence that the observed inhibition of CN by $H_2O_2$ could be the result of oxidative stress. Under these conditions (*e.g.* in the presence of $H_2O_2$ and pV(phen)), the cell viability was determined to be about 90%, and hence significant cytotoxic effects can be ruled out. Also, a strong

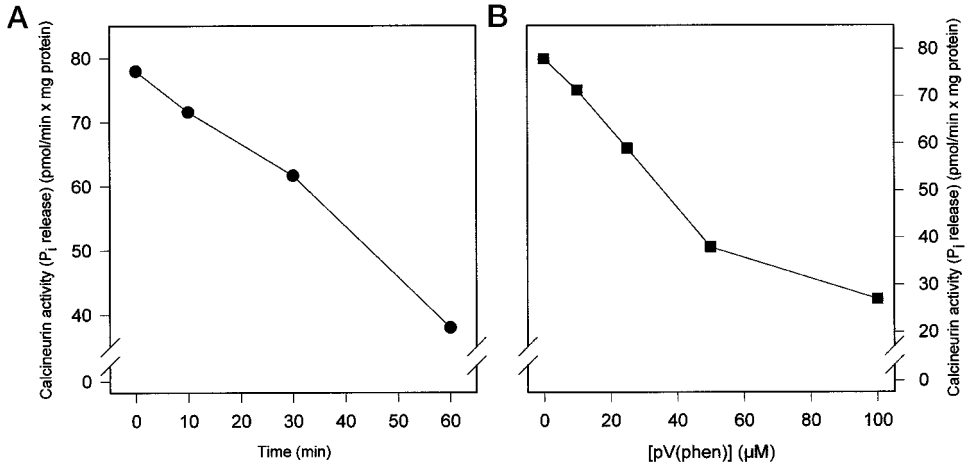


FIG. 7. **Inhibition of calcineurin activity in neutrophils incubated with pV(phen).** *A,* neutrophils ($7 \times 10^6$ cells/ml) were treated with 50 $\mu$M pV(phen) at 37 °C at different times. *B,* neutrophils ($7 \times 10^6$ cells/ml) were treated with different pV(phen) concentrations at 37 °C for 1 h. Calcineurin activity was assayed in cell lysates (80 $\mu$g of protein). Phosphatase activity is expressed as picomoles of phosphate released per minute per mg of protein. Means ± S.E. values from three separated experiments performed in triplicate are presented.

increase in the intracellular phosphotyrosine levels was detected in neutrophil lysates after incubation of the cells with pV(phen),[2] in agreement with previous reports on B lymphocytes (37).

EMSA analyses were carried out in order to test whether the changes in CN activity promoted by $H_2O_2$ were accompanied by an alteration of the DNA binding activity of two transcription factors, namely NF-AT and NF-κB. No binding activity was detected on neutrophil nuclear extracts when the NF-AT probe was used. However, activated NF-κB was detectable as a uniquely positioned band in assays of nuclear extracts from human neutrophils stimulated with LPS (1 $\mu$g/ml) (Fig. 8). On the basis of the results from McDonald *et al.* (46) and of supershift assays using anti-p50 and anti-p65 antibodies (data not shown), we interpreted the upper band as corresponding to the activated form of NF-κB (*i.e.* the p50/p65 tetramer). Gel retardation analysis of extracts from neutrophils stimulated with LPS and different doses of $H_2O_2$ demonstrated that $H_2O_2$ selectively inhibits the activation of NF-κB, resulting in a gradual decrease of the intensity of the p50/p65 band, as $H_2O_2$ was increased. As a negative control, we also analyzed the effect of CsA (1 $\mu$g/ml) (*lane 1*). In agreement with previous data from other cell lines (1–4), the p50/p65 band was only barely detectable in extracts from CsA/LPS-treated neutrophils. These results are consistent with an NF-κB activity regulated by CN in neutrophils, in agreement with previous reports (20–22). Present data concerning $H_2O_2$ inhibition of CN in neutrophils and lymphocytes suggest that previous evidence on $H_2O_2$-stimulated NF-κB functional activity (27, 29) can be interpreted as the result that peroxides, above a threshold level, are able to bypass the CN modulatory step and regulate transcription factors activity independently of CN.

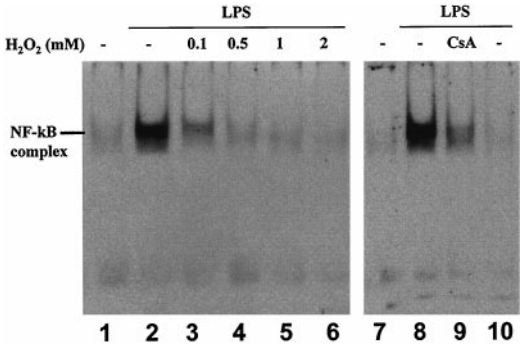


FIG. 8. **Reduced binding activity of NF-κB in $H_2O_2$-treated human neutrophils.** Binding activity of nuclear proteins (5 $\mu$g) from human neutrophils to a digoxigenin-labeled oligonucleotide containing the consensus NF-κB binding sequence was assessed by EMSA (see "Experimental Procedures"). The cells were untreated (*lanes 1* and *7*) or incubated with $H_2O_2$ (0.1, 0.5, 1, and 2 mM; *lanes 3*, *4*, *5*, and *6*, respectively) for 15 min prior to LPS stimulation (1 $\mu$g/ml) for 10 min. Before treatment with $H_2O_2$, cells were incubated with AMT (25 mM) for 30 min. CsA (1 $\mu$g/ml, *lane 9*) was added 30 min prior to LPS stimulation (1 $\mu$g/ml) without AMT. A control including a 100-fold excess of unlabeled oligonucleotide in the reaction performed with nuclear extracts from LPS-treated human neutrophils is also shown (*lane 10*). The figure shows a representative experiment out of three carried out with identical results.

## DISCUSSION

Only a few reports concerning CN in neutrophils and presenting indirect evidence of its presence in these cells have been published (23–26). All of them deal with neutrophil motility on vitronectin and its inhibition by CsA and FK 506 (23–26), a couple of well known inhibitors of CN activity. Here we have analyzed accurately, using a synthetic peptide corresponding to the phosphorylation site of the RII subunit of cyclic AMP-dependent protein kinase, the presence of CN in human neutrophils. Lysates from neutrophils dephosphorylate this substrate in a dose- and time-dependent manner. CN activity in neutrophils is insensitive to okadaic acid, whereas other phosphatases (*e.g.* 1A and 2A) are sensitive to this inhibitor (47). In the presence of okadaic acid the CN activity from neutrophil lysates is both $Ca^{2+}$-dependent and $Ca^{2+}$-sensitive to inhibition by trifluoroperazine, a calmodulin inhibitor. In addition, we present evidence that two well known immunosuppressants and inhibitors of CN activity in lymphocytes, CsA and FK 506 (1–4), potently depressed CN activity in neutrophils. It is well accepted that CN plays an important role as a prominent component of the calcium signaling pathway in T cells, by acting as an obligatory step between immunosuppressive drugs (*e.g.* CsA and FK 506) and some transcription factors (*e.g.* NF-AT and NF-κB) (1–4, 20, 21). The mechanism by which CN activates NF-AT seems rather complex. It has been shown that the coexpression in transfected cells of the activated CN and activated $p21_{ras}$ could mimic T cell receptor signaling during NF-AT induction, both acting as cooperative partners during T cell activation (52). Also, recent data show that CN forms a complex with cytosolic NF-AT4 (an isoform of NF-AT), which is transported to the nucleus where CN continues to dephosphorylate NF-AT4 (53). On the other hand the important role of ROI in the regulation of some transcription factors, mainly

NF-κB, is stressed by its activation in response to the addition of $H_2O_2$ (29), although doubts that it represents a universal phenomenon has been raised (30–32, 34). Moreover, an inverse relationship between ROI and NF-AT has also been pointed out, since low levels of $H_2O_2$ can actively suppress the transcription activity of NF-AT and the expression of interleukin-2 mRNA (35). These studies indicate that CN is a key component of the T cell signal transduction cascade and that oxidative signals can positively or negatively regulate transcription factor activity (54, 55). However, a deep knowledge of the molecular mechanism connecting both components (*i.e.* CN and oxidative signals) is lacking. We present here evidence for the first time that human neutrophils treated with $H_2O_2$ or pV(phen) exhibit a suppression of CN activity and that $H_2O_2$ effect required the previous inhibition of catalase activity. Only when catalase was inhibited by AMT a clear decrease of CN activity in the presence of exogenous $H_2O_2$ was observed. As expected, we have observed that the preincubation of neutrophils with the antioxidant PDTC cancelled the inhibition of CN activity by $H_2O_2$. As a preliminary effort toward the elucidation of the functional consequences of $H_2O_2$-dependent CN inhibition, we focused our attention on the transcription factor NF-AT as a potential target. However, we were unable to find any NF-AT DNA binding activity in nuclear extracts from neutrophils (data not shown). This fact closely agrees with previous results from immunoblotting analysis pointing out the absence of NF-AT proteins in neutrophils (56). Subsequent experiments were thus addressed to analyze whether $H_2O_2$-dependent CN inactivation could affect NF-κB activation. Conflicting results have been published on the presence or absence of NF-κB in human neutrophils. Browning *et al.* (57) did not observe any NF-κB activation in these cells. However, Cassatella and coworkers (46) described the presence of NF-κB subunits as well as the existence of NF-κB DNA binding activity in human neutrophils. We have followed the methodology described by the latter authors, with minor modifications, and have detected NF-κB DNA binding activity in fresh human neutrophils, together with its inhibition by $H_2O_2$. These data indicate that NF-κB activation is modulated by CN activity in human neutrophils.

Indirect evidence that CN is an enzyme sensitive to its redox environment has been reported, based on the fact that superoxide dismutase protects CN from spontaneous inactivation in brain crude extracts (50). This inactivation was interpreted as resulting from oxidative damage of the Fe-Zn active center of CN (50). Since there is a good evidence that CN is an Fe-Zn-containing enzyme (16), the hypothetical mechanism that can be proposed for this oxidative damage is that $H_2O_2$ and pV-(phen) could modify the redox state of the Fe-Zn center in the catalytic site and thereby inactivate the enzyme. This mechanism is in agreement with the observation that the reactivation of $H_2O_2$-inhibited neutrophil CN requires the addition of reducing agents, such as $Fe^{2+}$, ascorbate, and DTT (Fig. 6), as it was also previously demonstrated for a preparation of CN from brain (50). The observed inhibition of CN in intact neutrophils by oxidants, such as $H_2O_2$ and pV(phen), represents a novel mechanism of action for these agents. The pV(phen) molecule presents a dual activity, acting both as an intracellular oxidant and as an inhibitor of phosphotyrosine phosphatase (37). Evidence also has been presented that the activities of both protein tyrosine phosphatase and protein phosphatase 2A were reduced after $H_2O_2$ treatment of intact Jurkat T cells (58). Previously it has been described that CN has a regulatory phosphorylation site that is phosphorylated by the $Ca^{2+}$-independent form of calmodulin-kinase II. This phosphorylated CN exhibits a 50% decrease in its $V_{max}$ and 2-fold increase in the $K_m$ values (59). Thus, a hypothetical model in which oxidants modulate CN activity through phosphorylation of its regulatory site can therefore be postulated in the light of present data.

An apparent discrepancy between CN inhibition by $H_2O_2$ and other oxidant species, described here, and the protection exerted on CN by superoxide dismutase (50), which converts anion superoxide on $H_2O_2$, may be raised. In this context, however, the role of catalase and peroxidases, as detoxicant enzymes that degradate $H_2O_2$, should be introduced. These enzymes are also mutually protective, and therefore synergistic, when both $O_2^-$ and $H_2O_2$ are being made. In fact, experimental evidence (Fig. 4) illustrates the absence of effect by exogenous $H_2O_2$ added alone and the requirement of AMT to inhibit intracellular catalase and to detect $H_2O_2$-dependent CN inactivation. These data indirectly provide the notion of catalase as an additional protecting enzyme for CN against ROI inactivation. The implication of this suggestion is that, under normal physiological conditions, the cellular CN may be relatively less susceptible to molecular oxidative damage by ROI. Conversely, in pathological process, such as inflammation and reperfusion injury, or situations characterized by an inhibition of ROI-detoxicant enzymes, the CN inactivation by oxidant species may take place, and it could be a relevant process in the context of the oxidative stress state.

In summary, available evidence indicates that CN activity is a redox-sensitive step in cellular signaling cascades and suggests that the inhibition of NF-AT from lymphocytes (35) and NF-κB from neutrophils (present work) by $H_2O_2$ is elicited through inactivation of CN.

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